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Characterization and expression analysis of a gene encoding a secreted lipase-like protein expressed in the salivary glands of the larval Hessian fly, *Mayetiola destructor* (Say)

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ABSTRACT

In a salivary gland transcriptomics study we identified a cDNA with a full-length open reading frame for a gene (*MdesL1*) encoding a lipase-like protein expressed in the salivary glands of the larval Hessian fly, *Mayetiola destructor* (Say). Fluorescent *in situ* hybridization on salivary polytenes positioned *MdesL1* on the long arm of Autosome 1. BLASTp and conserved domain searches revealed the deduced amino acid sequence contained a lipase superfamily domain with similarity to lipases and phospholipases from other insects. A secretion signal peptide was identified at the amino terminus of the deduced amino acid sequence. Analysis of the transcript of *MdesL1* in larval Hessian fly tissues by quantitative real-time PCR (qPCR) revealed the greatest abundance was in salivary glands. Analysis of transcript levels during development showed the greatest level was detected in feeding 1st-instar and early 2nd-instar larvae. Transcript levels increased dramatically over time in larvae feeding on susceptible wheat but were detected at low levels in larvae feeding on resistant wheat. These data suggest the protein encoded by *MdesL1* is likely secreted into host-plant cells during larval feeding and could be involved in extra-oral digestion and changes in host-cell permeability or in generating a second messenger in a host-cell-signaling cascade.

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1. Introduction

The Hessian fly, *Mayetiola destructor* (Say), is a Cecidomyiid gall midge that is present in all of the wheat producing regions of the United States and poses a significant economic threat to wheat in terms of reduced grain yield (Ratcliffe et al., 2000; Smiley et al., 2004). Damage to wheat is entirely due to larval feeding and on seedling wheat this damage is manifested as increased leaf sheath permeability (Shukle et al., 1992), stunting and development of a dark green color in infested shoots and tillers, and, in heavy infestations, death of the plants (Byers and Gallun, 1972). To date, 32 Hessian fly resistance genes have been identified for protection of wheat (Sardesai et al., 2005), and the deployment of resistant cultivars in the United States has led to the development of genotypes of the pest that can overcome formerly resistant wheat within 5–10 years (Ratcliffe and Hatchett, 1997; Ratcliffe et al., 2000). Virulent genotypes (biotypes) of the Hessian fly pose a continuing threat to wheat production (Martin-Sanchez et al., 2003), and there is a need to identify alternative targets within the pest that could affect its ability to attack wheat.

The vital stain neutral red caused extensive staining of cells on the abaxial surface of the leaf when larvae fed on susceptible wheat versus small localized regions of staining in resistant wheat and no staining in non-infested wheat (Shukle et al., 1992; Subramanyam et al., 2007). These results indicate larval feeding on susceptible wheat increases the permeability of host cells within 24 h to neutral red as well as transport of the stain to adjacent cells. The rapid uptake of neutral red by plant cells has been shown to be a reliable indicator of gaps in the epicuticular/cuticular wax layers covering the cell wall with a correlation between stainability and the presence of such gaps (Joel and Juniper, 1982; Anderson, 2005). Thus, entry of neutral red into host cells in susceptible wheat due to larval feeding and transport to adjacent cells should be achieved through disruptions in the epicuticular/cuticular layers and cell wall, such as the small ruptures in the outer cell wall described by Harris et al. (2006), as well as disruption of the plasmalemma. Such

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disruptions could result from altered physiology of host cells and/or secretion of hydrolytic enzymes.

The altering of host physiology and development of nutritive tissue during larval Hessian fly attack of wheat is thought to be elicited through effector proteins in larval salivary secretions (Liu et al., 2004; Chen et al., 2004, 2006, 2008) in a manner analogous to the effector proteins reported for plant pathogens (Abramovitch and Martin, 2004, 2005; Grant et al., 2006). Additionally, the mandibles of the 1st-instar larval Hessian fly are minute blade-like structures that taper to a sharp-pointed tooth and appear to be adapted to piercing plant cell walls (Hatchett et al., 1990). Each mandible is grooved and contains a canal-like opening extending through the mandible, suggesting the opening could function in transport of salivary secretions into a host cell. The salivary glands connecting with the canal-like opening of the mandibles are composed of two regions; large basal cells that are polytene and a distal filamentous region of polyploid cells (confer Grover et al., 1988), with the basal salivary gland cells thought to be the primary source of salivary secretions in 1st- and early 2nd-instar larvae.

Lipids and phospholipids are major components of cell membranes, and various secreted lipases and phospholipases have been hypothesized to be involved in the membrane disruption processes that occur during microbial infection and have been included among the virulence factors required for infection by various microbial pathogens (Salyers and Wit, 1994; Comménil et al., 1995, 1998, 1999; Ghannoum, 2000; Nasser Eddine et al., 2001; Voigt et al., 2005). Phospholipases also play a pivotal role in many signaling cascades regulating critical cellular functions in animals and plants (Meldrum et al., 1991; Munnik et al., 1995; Shortridge and McKay, 1995; Wang, 1999; Rose and Prestwich, 2006). Additionally, cutinases are lipolytic enzymes of the α/β-hydrolase fold family to which other lipases belong (Carvalho et al., 1998; Longhi and Cambillau, 1999) and are associated with the attack of plants by various pathogens. As digestive enzymes, lipases, and phospholipases have been associated with the midgut contents of several insect species (Uscian et al., 1995; Nor Aliza and Stanley, 1998; Rana et al., 1998; Nor Aliza et al., 1999) as well as in salivary gland secretions (Ribeiro and Francischetti, 2001; Valenzuela et al., 2003; Ribeiro et al., 2004; Tunaz and Stanley, 2004; Oliveira et al., 2006; Sarker and Mukhopadhyay, 2006; Wooding et al., 2007).

We have a continuing interest in the digestive enzymes of the larval Hessian fly because of their possible involvement in damage to the host plant during feeding. Through an analysis of the salivary transcriptome of the Hessian fly we identified the transcript of a gene (designated *MdesL1*) that encoded a secreted lipase-like protein. We were interested in a gene encoding a secreted lipase-like protein because of a possible role in the early phase of the interaction of larvae with wheat and the reported increased permeability and cytological changes in host-plant cells. Specific objectives of the present study were to: (1) characterize the gene *MdesL1*; (2) determine the spatial patterns of transcript levels of *MdesL1* in larval tissues as well as the temporal patterns during development; (3) assess the transcript levels of *MdesL1* in larvae feeding on susceptible and resistant wheat.

2. Materials and methods

2.1. Insect and plant material

Hessian fly Biotype L used in the present study was maintained in culture under greenhouse condition on wheat cultivar 'Magnum' (carries resistance gene *H5*). Biotype L was established in culture from a field collection of Hessian fly made in Posey County, Indiana in 1986. Two near isogenic wheat lines 'Newton' (carries no genes for resistance) and 'Iris' (carries resistance gene *H9*) (Patterson et al., 1994) were used to provide compatible and incompatible Hessian fly–wheat interactions. Biotype L on susceptible Newton wheat represented a compatible interaction, and Biotype L on resistant Iris wheat represented an incompatible interaction.

2.2. Insect life stages, larval dissections, collection of larvae in compatible/incompatible interactions, and RNA extraction

To determine transcript levels of *MdesL1* during development, 1st-, 2nd-, and 3rd-instar larvae and pupae were collected from susceptible Newton wheat in 1.5-ml micro-centrifuge tubes by dissecting the crown portion of plants and immediately flash-frozen in liquid nitrogen and stored at −80 °C. For 1st- and 2nd-instar larvae, a volume of 30–50 μl was collected per 1.5-ml micro-centrifuge tube. To estimate the number of larvae collected, the volume of individual larvae was calculated as that of a cylinder. For early 1st-instar larvae this equated to approximately 1600 individulas/30 μl, and for early 2nd-instar larvae approximately 200 individuals/50 μl. For 3rd-instar larvae and pupae approximately 80 individuals were collected per 1.5-ml micro-centrifuge tube. For adults, approximately 70 individuals were collected after emergence, cold anesthetized, placed in 1.5-ml micro-centrifuge tubes, and flash-frozen in liquid nitrogen. Total RNA was extracted from larvae, pupae, and adults using the RNAqueous-4PCR™ kit from Ambion (http://www.ambion.com) following the manufacturer's protocol and stored at −80 °C until utilized. To evaluate transcript levels in larval tissues, approximately 300 of each tissue evaluated, midguts, salivary glands, and fat bodies, were dissected as previously described (Grover et al., 1988; Mittapalli et al., 2005) from late 1st-instar larvae (96-h old). The dissected tissues were collected directly in 100 μl of lysis buffer from the RNAqueous-4PCR™ kit contained in a 1.5 ml micro-centrifuge tube, and RNA was isolated immediately after collection using the RNAqueous-4PCR™ kit protocol. To assess transcript levels during compatible and incompatible interactions 1st-instar larvae feeding on either susceptible Newton (compatible interaction) or resistant Iris (incompatible interaction) wheat were collected and frozen as described above at 24, 48, 72, and 96 h after they had infested the plants. RNA was extracted from larvae undergoing compatible and incompatible interactions as described above. Two biological replicates, separated temporally, were included for the compatible/incompatible interaction study.

2.3. Recovery of cDNA and genomic clones of MdesL1 from Hessian fly

cDNAs of *MdesL1* were recovered by reverse transcription PCR (RT-PCR) with the SuperScript™ One Step RT-PCR System and Platinum® *Taq* DNA Polymerase High Fidelity (Invitrogen, http://www.invitrogen.com) using primers (Table 1) designed to the ends

Table 1
PCR primer sequences.

	Primer sequence 5′–3′	T_m (°C)
cDNA primers		
*MdesL1*cDNA-F	GACACAAAAGTGAAAAATGTTAGGC	54.7
*MdesL1*cDNA-R	CGTTTTAAATAATTTATTTTTTGAATTTATTTG	48.8
qPCR primers		
Target gene		
*MdesL1*qPCR-F	AGCCGTGCGCAAGAAGAA	58.6
*MdesL1*qPCR-R	AATGAAGACTTGTTGCCGAATTC	55.2
Internal reference		
*MdesUB1*qPCR-F	CCCCTGCGAAAATTGATGA	54.5
*MdesUB1*qPCR-R	AACCGCACTACTTGCATCGAA	56.7

of the 5′,3′ untranslated regions of a cDNA encoding a lipase-like protein recovered from a larval Hessian fly salivary gland EST library (M.-S. Chen, personal communication) and 1.0 μg total RNA extracted from 1st-instar Biotype L larvae (96-h old) as the template. Genomic clones of *MdesL1* were recovered by standard PCR using DNA extracted from Biotype L adults with the DNeasy® Tissue Kit (Qiagen, http://www1.qiagen.com) and the primers used to recover cDNA clones (*vide supra*). PCR fragments of cDNA and genomic sequences were cloned in the pCR®4-TOPO® Vector using the TOPO TA Cloning® Kit (Invitrogen, http://www.invitrogen.com).

2.4. Sequencing and sequence analysis

Three independent clones of each cDNA/DNA fragment were sequenced using both strands and M13 forward and reverse primers by the Purdue Genomics Center. Consensus sequences were obtained using the alignment consensus programs CONS (creates a consensus from multiple alignments) and MERGER (merges two overlapping sequences) (available in EMBOSS through the Purdue Genomics Center (http://pro.genomics.purdue.edu/emboss/). Sequence similarity/annotations and analysis for conserved domains were performed using BLAST programs on the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov). Secretion signal peptide analysis with the deduced amino acid sequence of the lipase-like protein was performed using SignalP 3.0 (Bendtsen et al., 2004; http://www.cbs.dtu.dk/services/SignalP/) and molecular weight was determined with Protein Calculator V3.3 (http://www.scripps.edu/~cdputnam/protcalc.html).

For phylogenetic analyses, amino acid sequences were aligned using ClustalX (1.81) software (Thompson et al., 1997). PAUP 4.0b10 for Windows (Swofford, 1998) was used to conduct parsimony and distance/neighbor-joining analyses. A secreted phospholipase from *Homo sapiens* (NP_110448) was used as the outgroup. For parsimony analysis, starting tree(s) were obtained via stepwise addition with the tree bisection-reconnection branch-swapping algorithm. The distance/neighbor-joining analysis (Saitou and Nei, 1987) used the total number of pairwise character differences (TOTAL) as the distance setting. Confidence values for the groupings in the trees were assessed by bootstrap resampling (Felsenstein, 1985) with 1000 repetitions. Gaps were excluded from the analyses.

2.5. Quantitative real-time PCR (qPCR) and statistical analysis

Total RNA extracted from isolated tissues, developmental stages, or larvae during compatible and incompatible interactions was used to generate template ssDNA for qPCR using the SuperScript™ III First-Strand Synthesis System (Invitrogen, http://www.invitrogen.com). The software Primer Express from Applied Biosystems (http://www.appliedbiosystems.com/) was used to design the qPCR primers. Relative expression analyses were performed using a Hessian fly *ubiquitin* gene transcript as the internal reference. Quantification of mRNA levels, displayed as relative expression values (REV), was based on the Relative Standard Curve method (Applied Biosystems Instruments User Bulletin #2: ABI Prism 7700 Sequence Detection System http://hcgs.unh.edu/protocol/realtime/UserBulletin2.pdf). Primer sequences for the Hessian fly *lipase-like* and *ubiquitin* transcripts used in qPCR analyses are referenced in Table 1.

For calculations of significance, the logs of the REVs for each gene were analyzed by analysis of variance (ANOVA) using the PROC MIXED procedure of SAS (SAS Institute Inc. SAS/STAT User's Guide, Version 9.1). For analysis of expression in tissues and developmental stages, the statistical model included treatment and interaction between treatments, whereas for the analysis of expression in different interactions (compatible and incompatible), the statistical model included treatment, time points, and interaction between treatments and time points as fixed effects. Biological replicates were included as a random effect in the analysis model. Treatment differences at each time point were evaluated using orthogonal contrasts and considered statistically significant if the *p*-value associated with the contrast was $p < 0.05$ (Yuan et al., 2006).

2.6. Fluorescent in situ hybridization (FISH) on Hessian fly salivary polytenes

Polytene chromosomes were isolated from salivary glands of early 2nd instars and prepared as described by Shukle and Stuart (1995). FISH was performed as described by Chen et al. (2004). In brief, the probe was prepared by labeling a recovered genomic clone of *MdesL1* (*vide supra*) with biotin by nick translation. Detection was performed using Alexa Fluor 488-conjugated strepavidin (Invitrogen/Molecular Probes, http://www.invitrogen.com/site/us/en/home/brands/Molecular-Probes.html). Chromosomes were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) in VECTASHIELD mounting medium for fluorescence (Vector Laboratories, http://www.vectorlabs.com/).

3. Results

3.1. Characterization of MdesL1

The genomic sequence of *MdesL1* from Hessian fly was submitted to GenBank and was assigned the accession no. EU877196. The recovered cDNA for *MdesL1* was 1160 bp in length and had an open reading frame (ORF) of 978 bp that encoded 326 amino acids. The ORF was judged full-length because of the presence of an inframe stop codon (TGA) at position −6 in the 5′ untranslated region preceding the ATG translation initiation codon. The genomic sequence for the coding region was 1294 bp in length, and the genomic coding region consisted of five exons separated by four introns. The five exons varied in length from 83 bp (exon 4) to 425 bp (exon 5), and the four introns varied in length from 62 bp (intron 2) to 112 bp (intron 4). The hexanucleotide consensus polyadenylation signal sequence AATAAA seen in other species preceded the polyA tail by 30 bp.

Sequence similarity and annotation searches using the BLAST (blastp) program on the NCBI website with the deduced amino acid sequence for *MdesL1* revealed similarity to a number of lipases and phospholipases from insects. For example, these included a lipase from *Bombus ignitus* Smith (ABY84699) with 45% positives (7e−11), and a phospholipase A_1 precursor from *Vespula vulgaris* (L.) (P49369) with 42% positives (4e−10) at the amino acid level amongst others. A conserved domain search on the NCBI website identified a lipase superfamily/pancreatic lipase-like conserved domain (*E*-value 2e−12) containing a catalytic triad and nucleophilic elbow in the Hessian fly protein MdesL1. Alignment of MdesL1 with insect lipases and phospholipases further revealed a putative catalytic triad (Cys-160, Asp-188, and His-252), a nucleophilic elbow (Gly-158, X-159, X-161, Gly-162), and an active site flap/lid (Cys-241, X-242, X-248, Cys-249) (Fig. 1). A secretion signal peptide (MLGIKLFFVVVFFVGLNG) with a predicted cleavage site between Gly-18 and Gln-19 was identified at the amino terminus of the lipase-like protein MdesL1 using the SignalP 3.0 server. Molecular weight for MdesL1 including the secretion signal peptide was calculated as 37.4 kD.

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M. destructor    GIDG--DYAQYIQYYDGIKLRVPYVIETLTNFIGRLKKFALIKSANQITAAGFCLGGHMA 166
V. vulgaris      AACTNEAAGLKYLYYPTAARNTRLVGQYIATITQKLVKHYKIS-MANIRLIGHSLGAHAS 179
P. paulista      GACNAFASTLDYLGYSTAVGNTRHVGKYVADFTKLLVEQYKVS-MSNIRLIGHSLGAHTS 162
A. gambiae       GAST--------LLYPVARYRVPKVANLVAALIDNLVAGLGQD-INQIGIIGHSLGAHIA 225
                 .             *     ..  * : :: :   *      .   :*   *..**.* :

M. destructor    GMLGRDVEYLFGEKIRMVLAFDPPKYGFERNSLMFRERVQKGDAKYVEVTHTS--FIGMY 224
V. vulgaris      GFAGKKVQELKLGKYSEIIGLDPARPSFDSN--HCSERLCETDAEYVQIIHTS-NYLGTE 236
P. paulista      GFAGKEVQELKLNKYSNIDGLDPAGPSFDSN--DCPERLCETDAEYVQIIHTS-NILGVY 219
A. gambiae       GIAAKRVRSGKIG---YIVGLDPASPLFRLK--KPDERLSADDAQYVEIIHTNGKALGFF 280
                 *: .: *.         : .:**.   *  :     **:   **:**:: **.   :*

M. destructor    TNDADTDLILNN-LRQPGCPHDFVDFCNHNAALFFHQHIFMHTKPAP--------FLASK 275
V. vulgaris      KTLGTVDFYMNNGKNQPGCGRFFSEVCSHSRAVIYMAECIKHECCLIGIPKSK---SSQP 293
P. paulista      SKIGTVDFYMNYGSHQPGCGRFFSPSCSHTKAVKYLTECIKHECCLIGTPWKKYFSTPKP 279
A. gambiae       SNIGQADYYPNGGVRQPGCG--FSLTCSHQRAVDFFKESLKISNYYA----RRCDGIANL 334
                 .. . .*   *   .****   *   *.*  *: :  . :                 ..
```

Fig. 1. Multiple sequence alignment of the lipase superfamily/pancreatic lipase-like conserved domain of MdesL1 (EU877196) showing homology with the conserved domains of a venom phospholipase A_1 from *Vespula vulgaris* (P49369), a venom phospholipase A_1 from *Polybia paulista* (A2VBC4), and a pancreatic-lipase-like enzyme from *Anopheles gambiae* (XP_318018). Catalytic triad residues are shown in red, nucleohilic elbow residues are depicted in green, and active site flap/lid residues are displayed in orange. Identical residues among all taxa are indicated by '*', conserved substitutions by ':' and semi-conserved substitutions by '.' symbols.

3.2. Phylogenetic analysis and chromosomal localization of MdesL1

A phylogenetic analysis of the deduced Hessian fly protein MdesL1 with secreted (i.e., signal peptide predicted) lipases and phospholipases from other insects using the parsimony and distance/neighbor-joining methods yielded trees with congruent topology. Both the deduced lipase-like protein from the Asian rice gall midge, *Orseolia oryzae* (Wood-Mason), and the deduced lipase-like protein MdesL1 from Hessian fly fell basally within a large main clade (clade 3) (Fig. 2). Two other subclades within the main

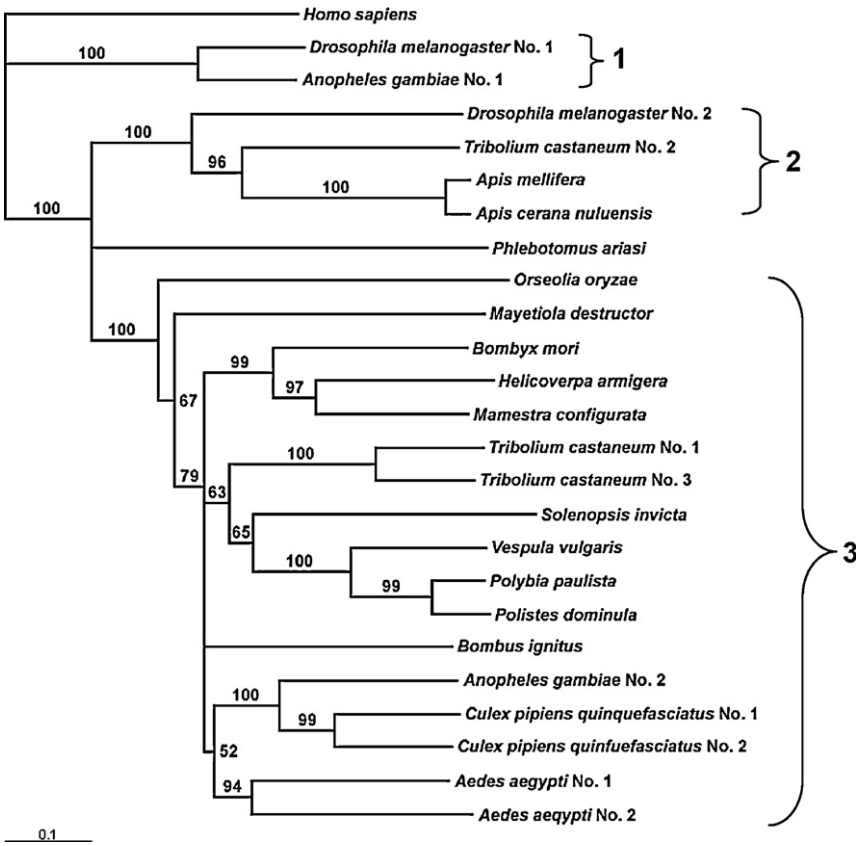

Fig. 2. Dendrogram of Hessian fly lipase-like protein MdesL1 and secreted lipases and phospholipases from other insects. The dendrogram was calculated from aligned amino acid sequences and produced by the distance/neighbor-joining criteria. A phospholipase A_2 from *Homo sapiens* was used as the outgroup. Numbers at the nodes represent the percentage of 1000 bootstrap replications supporting that node. Maximum parsimony yielded congruent topology and support. The Hessian fly, *Mayetiola destructor*, lipase-like protein MdesL1 fell basal in the large clade containing a number of secreted lipases, phospholipases, and venom phospholipases from other insects. GenBank accession nos. are *Homo sapiens* (NP_110448), *Drosophila melanogaster* No. 1 (NP_648815), *Anopheles gambiae* No. 1 (XP_309537), *Drosophila melanogaster* No. 2 (NP_724556), *Tribolium castaneum* No. 2 (XP_971093), *Apis mellifera* (NP_001011614), *Apis cerana nuluensis* (A59055) *Phlebotomus ariasi* (AAX54852), *Orseolia oryzae* (FJ196713), *Mayetiola destructor* (EU877196), *Bombyx mori* (NP_00104159), *Helicoverpa armigera* (ABK29468), *Mamestra configurata* (ACD37365), *Tribolium castaneum* No. 1 (XP_971476), *Tribolium castaneum* No. 3 (XP_971532), *Solenopsis invicta* (AAT95008), *Vespula vulgaris* (P49369), *Polybia paulista* (A22VBC4), *Polistes dominula* (Q6Q252), *Bombus ignitus* (ABY84699), *Anopheles gambiae* No. 2 (XP_318018), *Culex pipiens quinquefasciatus* No. 1 (XP_001853508), *Culex pipiens quinquefasciatus* No. 2 (XP_001853511), *Aedes aegypti* No. 1 (XP_001652528),*Aedes aegypti* No. 2 (XP_001652527).

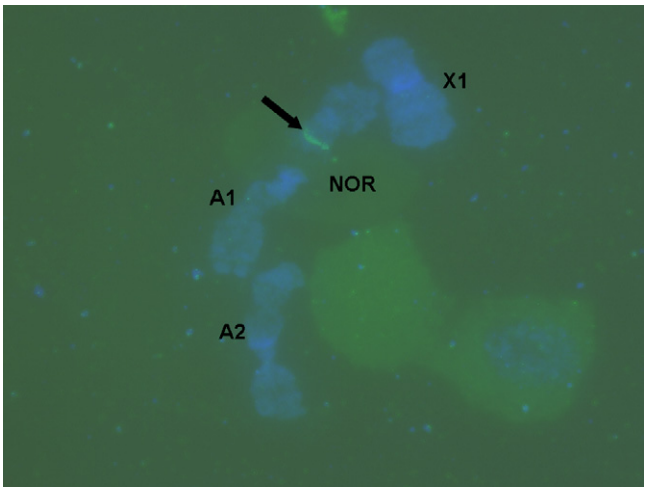

Fig. 3. Results of fluorescent *in situ* hybridization (FISH) of *MdesL1* on Hessian fly, *Mayetiola destructor*, salivary polytene chromosomes. Arrow indicates the hybridization signal for *MdesL1* (green fluorescence) observed on Autosome 1 (A1). The signal for *MdesL1* was observed on both chromatids of the long arm of A1 distal to the nucleolar organizing region (NOR). No hybridization signals were observed on Autosome 2 (A2) or the sex chromosomes X1 and X2. Chromosome X2 was outside the presented field of view and the image is not shown.

clade contained secreted lipases and phospholipases from Lepidoptera/Diptera and Hymenoptera/Coleoptera, respectively. Secreted phospholipases from *Drosophila melanogaster* Meigen, *Anopheles gambiae* Giles, *Tribolium castaneum* (Herbst), *Apis mellifera* L. and *Apis cerana nuluensis* Tingek, Koeniger & Koeniger, respectively comprised two small clades (clades 1 and 2).

To determine the chromosomal location of *MdesL1*, fluorescent *in situ* hybridization (FISH) was performed on salivary gland polytene chromosomes. Results gave a single hybridization signal located on the long arm of Autosome 1 (A1) near the nucleolar organizing region (NOR) (Fig. 3).

3.3. Abundance of MdesL1 transcripts in larval tissues, life stages, and during interactions with wheat

Analysis of the spatial distribution of *MdesL1* transcripts in fat bodies, salivary glands, and midgut of late 1st-instar larvae by qPCR revealed the transcript level was significantly greater ($p < 0.05$) in salivary glands (Fig. 4). The lowest level of transcript detected was in the midgut tissue, and thus, the levels of transcript in salivary gland and fat body tissues were compared relative to the

Fig. 4. Transcript levels of the Hessian fly, *Mayetiola destructor*, *MdesL1* gene in tissues of virulent Biotype L larvae on susceptible Newton wheat. Mean relative expression values (REVs) are plotted for the *MdesL1* transcript in fat bodies, salivary glands, and midgut. REVs were calculated relative to the expression of a Hessian fly ubiquitin gene. Error bars represent the standard error for three technical replicates. Transcript level in salivary glands was significantly greater ($p < 0.05$) compared to fat bodies and midgut.

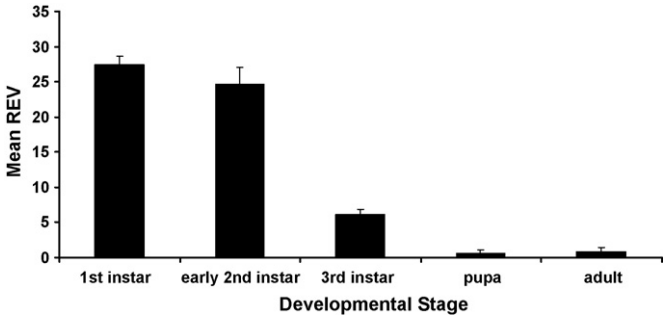

Fig. 5. Transcript levels of the Hessian fly, *Mayetiola destructor*, *MdesL1* gene during development of virulent Biotype L larvae on susceptible Newton wheat. Mean relative expression values (REVs) for the *MdesL1* transcript are plotted for 1st instar, early 2nd instar, 3rd instar, pupa, and adult. REVs were calculated relative to the expression of a Hessian fly ubiquitin gene. Error bars represent the standard error for three technical replicates. Transcript levels in 1st- and early 2rd-instar larvae were significantly greater ($p < 0.05$) compared to the other stages in development.

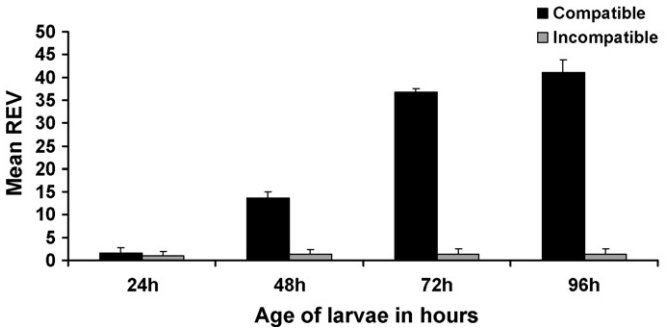

Fig. 6. Transcript levels of the Hessian fly, *Mayetiola destructor*, *MdesL1* gene during compatible and incompatible interactions with wheat. Analysis was conducted with RNA isolated from larvae in compatible (Biotype L larvae on susceptible 'Newton' wheat) and in incompatible interactions (Biotype L larvae on resistant 'Iris' wheat) at 24 h post-hatch through 96 h post-hatch. Mean relative expression values (REVs) for the *MdesL1* transcript are plotted for each of the four time points. REVs were calculated relative to the expression of a Hessian fly ubiquitin gene. Error bars represent the standard error for two biological replicates with two technical replicates within each. Transcript levels for larvae in compatible interactions were significantly greater than for larvae in incompatible interactions ($p < 0.05$) at 48, 72, and 96 h.

basal level in midgut. The level of transcript of *MdesL1* was 92-fold greater in salivary gland tissue relative to midgut tissue, while in fat bodies the transcript level was almost equivalent to the level detected in midgut (i.e., 1.1-fold relative to midgut tissue). During development on susceptible Newton wheat (larvae do not develop on resistant wheat and die in 5–6 days) the transcript of *MdesL1* was detected in all life stages. However, the transcript levels were significantly greater ($p < 0.05$) in 1st- and 2nd-instar larvae compared to the other life stages with the lowest level of transcript detected in pupae (Fig. 5). Relative to pupae, the *MdesL1* transcripts in 1st-, 2nd-, 3rd-instar larvae, and adults were 94-fold, 84-fold, 10.3-fold, and 1.5-fold greater, respectively. The temporal patterns of *MdesL1* transcript were also determined in 1st-instar larvae feeding on susceptible Newton wheat and resistant Iris wheat. Significantly greater ($p < 0.05$) levels of *MdesL1* transcript were detected in larvae feeding on susceptible Newton wheat 48, 72, and 96 h post-hatch compared to larvae feeding on resistant Iris wheat (Fig. 6).

4. Discussion

Despite its economic importance as a pest of wheat, only recently have the interactions between the Hessian fly and wheat

begun to be revealed at the biochemical and molecular levels. In particular, how Hessian fly larvae elicit the changes and re-programming in host-plant tissues that result in the establishment of a nutritive tissue layer to feed the developing larvae is not well understood. Here we report on the characterization and expression analysis of a Hessian fly gene, *MdesL1,* encoding a lipase-like protein expressed in larval salivary glands and hypothesize its possible role in the establishment of a feeding site and cytological changes in host tissues associated with larval feeding.

Characterization of the genomic and cDNA sequences of *MdesL1* indicated a full-length genomic coding region had been recovered. BLAST (blastp) similarity and annotation searches with the deduced amino acid sequence of *MdesL1* identified a lipase superfamily/pancreatic lipase-like conserved domain and revealed similarity to a number of lipases and phospholipases from other insects. While the similarity to various other lipases/phospholipases was not highly significant, the lipases and phospholipases in general comprise a very heterogeneous group of enzymes, and the level of similarity seen here supports *MdesL1* encoding a lipase-like protein.

The calculated molecular weight for the protein MdesL1 was 37.4 kD, which is similar to that for other lipases and phospholipases: a phospholipase A_1 from *V. vulgaris* (P49369) 37.7 kD, a phospholipase A_2 from *An. gambiae* (XP_309537) 43.2 kD, a lipase from *Helicoverpa armigera* (Hübner) (ABK29468) 31.1 kD, and a lipase from *Aedes aegypti* (L.) (XP_001652528) 39 kD. Within the conserved domain identified in MdesL1 a putative catalytic triad, a nucleophilic elbow, and an active site flat/lid were identified, which shared similarity in residues to those seen in other lipases/phospholipases. Surprisingly, the putative catalytic triad identified had a cysteine thiol in the catalytic triad (Cys-160) rather than a serine residue as is the case in most lipases/phospholipases. However, the phospholipase Ds have conserved His, Lys, and Asp residues that form the catalytic triad responsible for catalysis (Sung et al., 1997; Wang, 1999). It is also interesting to note that a transcript coding for a secreted salivary lipase-like protein has been identified in the salivary gland transcriptome of the Asian rice gall midge. However, the lipase-like protein from the Asian rice gall midge had a serine in its putative catalytic triad instead of the cysteine found in the Hessian fly protein MdesL1 (O. Mittapalli and R.H. Shukle, unpublished data). The implications for the cysteine substitution for serine in the catalytic triad of MdesL1 remains to be pursued in future studies.

Results of the phylogenetic analysis gave additional support to the annotation of MdesL1 as a secreted lipase-like protein, and its basal position along with that of the putative lipase-like protein from the Asian rice gall midge could indicate these two deduced proteins are evolutionarily older than those positioned toward the terminal nodes of the dendrogram. All of the sequences included in the dendrogram were secreted lipases and phospholipases from insects and the three main groupings and subgroups within these groups formed due to similarities among their aligned amino acid sequences that in some cases could reflect functional relationships. In this regard, it is interesting to note that the venom phospholipases from *Solenopsis invicta* Buren, *Vespula vulgaris* (L.), *Polybia paulista* Ihering, and *Polistes dominula* Christ grouped together as did the pancreatic lipase-like enzymes from *Bombyx mori* L., *H. armigera*, and *Mamestra configurata* Walker, while the bee-venom-like phospholipases from *D. melanogaster* and *T. castaneum* grouped with the venom phospholipases from *A. mellifera* and *A. cerana nuluensis*.

Results from FISH revealed the chromosomal location of *MdesL1* (long arm of Autosome 1, A1) and suggested it is present as a single copy in the Hessian fly genome. While the novel secreted salivary gland proteins identified by Chen et al. (2004, 2006) are distributed throughout the genome of the Hessian fly (J.J. Stuart and M.-S. Chen, personnel communication) it is of interest to note that the genes encoding three of these secreted salivary proteins (11A1, 11C1, and 11B1) are also located on the long arm of A1 with the chromosomal location of 11B1 (Chen et al., 2006) close to that shown here for *MdesL1*.

Transcript levels for MdesL1 in larvae on susceptible wheat were significantly greater than in larvae on resistant wheat. We speculate this results from incompatibility on resistant wheat and is in agreement with the observation that larvae infesting resistant wheat are unable to establish a feeding site for normal development and die in 5–6 days post-hatch (Painter, 1930).

The mandibles of the larval Hessian fly are well adapted to channel salivary secretions into host-plant cells, and lipases have been well documented in salivary secretions and extra-oral digestion of phytophagous heteropterans (Oliveira et al., 2006; Sarker and Mukhopadhyay, 2006; Wooding et al., 2007). Further, the rapid and extensive increase in the permeability of host-plant cells during larval feeding (Shukle et al., 1992; Subramanyam et al., 2007), the development of small gaps in the walls of host epidermal cells, and the breakdown of organelles and movement of cytoplasm from compromised cells into adjacent cells devoid of cytoplasm (Harris et al., 2006) are supportive of the actions of a hydrolytic enzyme such as a lipase or a phospholipase playing a role in the feeding of larval Hessian fly on its host plant.

Given that MdesL1 showed similarity to phospholipases and, in particular, venom phospholipases from various insects in blastp searches it is plausible that it has similar roles in larval Hessian fly. Phospholipases, through their hydrolysis of phospholipids, not only impact cellular membrane integrity but also generate second messengers that are involved in cell-signaling cascades in both animals and plants (Munnik et al., 1995; Wang, 1999; Rose and Prestwich, 2006). How Hessian fly larvae re-program the host-plant tissues at their feeding sites toward development of a nutritive tissue is very intriguing but unknown. To date, a large number of families of novel (do not show similarity to other entries in protein databases) secreted salivary gland proteins have been identified through an extensive salivary gland EST project (Liu et al., 2004; Chen et al., 2004, 2006, 2008). It has been speculated that these novel secreted salivary gland proteins are effectors involved in the re-programming of host tissues and the dramatic altering of host carbon and nitrogen metabolism (Zhu et al., 2008). Thus, it is conceivable that the protein encoded by *MdesL1* characterized in the present study could be an effector enzyme that catalyzes the formation of a second messenger regulating a cell-signaling cascade in host-plant cells contributing to the re-programming of host tissues.

The focus of the present study was to characterize *MdesL1* and investigate the abundance of its transcript spatially in larval tissues, temporally during development, and in larvae during compatible and incompatible interactions with wheat. Results from these studies support *MdesL1* as encoding a secreted salivary hydrolytic enzyme lipase-like in nature that we hypothesize is involved in the establishment of larval feeding sites and perhaps changes in host-cell permeability. The presence of genes encoding secreted salivary lipase-like enzymes in both the Hessian fly and the Asian rice gall midge may indicate such hydrolytic enzymes are involved in the establishment of larval feeding sites and feeding in other Cecidomyiid species. Future studies will be directed toward proteomic and RNAi functional genomic approaches to reveal the role of *MdesL1* in the interaction between larval Hessian fly and wheat. Preliminary results have revealed that extract of salivary glands from late 1st- and early 2nd-instar Hessian fly larvae has lipase activity in a tritrimetric assay using olive oil as the substrate (R.H. Shukle and A.J. Johnson, unpublished data). Whether the

activity detected was due to MdesL1 described in the present study or another lipase is unknown and will be addressed in future proteomic studies. To date, expression of MdesL1 in an *Escherichia coli* cell system has resulted in the expressed protein residing entirely in the inclusion bodies and not refolding properly after being solubilized. These results may indicate a different cell expression system, such as an insect cell system, is required for expression of MdesL1 in a soluble fraction.

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